(12) # EUROPEAN PATENT APPLICATION

(54) Cysteine-depleted muteins of interferon-beta, their preparation, formulations containing them, and structural genes, vectors and organisms, and their production, suitable for use in the preparation of said muteins.

(57) The invention provides muteins of the biologically active proteins of IFN-β in which cysteine residues that are not essential to biological activity have been deleted or replaced with other amino acids to eliminate sites for intermolecular crosslinking or incorrect intramolecular disulfide bridge formation. These muteins may be made via bacterial expression of mutant genes that encode the muteins and have been synthesized from the genes for the parent proteins by oligonucleotide-directed mutagenesis.

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CYSTEINE-DEPLETED MUTEINS OF INTERFERON-BETA, THEIR
PREPARATION, FORMULATIONS CONTAINING THEM, AND
STRUCTURAL GENES, VECTORS AND ORGANISMS, AND THEIR
PRODUCTION, SUITABLE FOR USE IN THE PREPARATION OF
SAID MUTEINS

5   This invention is in the general area of
recombinant DNA technology. More specifically it
relates to mutationally altered biologically active
Interferon-beta proteins that differ from their
parent analogs by substitution/deletion of cysteine
residues.
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           Biologically active proteins that are micro-
bially produced via recombinant DNA (rDNA) technology
may contain cysteine residues that are nonessential to
their activity but are free to form undesirable inter-
15 molecular or intramolecular links. One such protein
is microbially produced human beta interferon
(IFN-ß). In the course of the preparation of IFN-ß by
rDNA techniques, it has been observed that dimers and
oligomers of microbially produced IFN-ß are formed in
20 E.coli extracts containing high concentrations of
IFN-ß. This multimer formation renders purification
and separation of IFN-ß very laborious and time-
consuming and necessitates several additional steps in
purification and isolation procedures such as reducing
25 the protein during purification and reoxidizing it to
restore it to its original conformation, thereby
increasing the possibility of incorrect disulfide bond
formation. Furthermore, microbially produced IFN-ß
has also been found to exhibit consistently low
30 specific activity due perhaps to the formation of
multimers or of random intramolecular disulfide

bridges. It would be desirable, therefore, to be able to alter microbially produced biologically active proteins such as IFN-ß in a manner that does not affect their activity adversely but reduces or eliminates
5   their ability to form intermolecular crosslinks or intramolecular bonds that cause the protein to adopt an undesirable tertiary structure (eg, a conformation that reduces the activity of the protein).

The present invention is directed to producing by directed mutagenesis techniques mutationally altered biologically active IFN-ß proteins (such proteins are called "muteins", Glossary of Genetics and Cytogenetics, 4th Ed, p 381, Springer-Verlag (1976)) that retain the activity of their parent analogs but lack the ability to form intermolecular links or undesirable intramolecular disulfide bonds. In this regard Shepard, H.M., et al, Nature (1981) 294:563-565 describe a mutein of IFN-ß in which the cysteine at position 141 of its amino acid sequence (there are three cysteines in native human IFN-ß at positions 17, 31, and 141, Gene (1980) 10:11-15 and Nature (1980) 285:542-547) is replaced by tyrosine. This mutein was made by bacterial expression of a hybrid gene constructed from a partial IFN-ß cDNA clone having a G ⟶ A transition at nucleotide 485 of the IFN-ß gene. The mutein lacked the biological activity of native IFN-ß leading the authors to conclude that the replaced cysteine was essential to activity.

Directed mutagenesis techniques are well known and have been reviewed by Lather, R.F. and Lecoq, J.P. in Genetic Engineering Academic Press (1983) pp 31-50. Oligonucleotide-directed mutagenesis is specifically reviewed by Smith, M. and Gillam, S. in Genetic Engineering: Principles and Methods, Plenum Press (1981) 3:1-32.

The invention provides a biologically active recombinant synthetic interferon-beta mutein in which the cysteine residue normally present at position 17, numbered in accordance with nature human interferon-beta, and which is free to form a disulfide link and is non-essential to said biological activity has been deleted or replaced by another amino acid.

Another aspect of the invention are synthetic structural genes having DNA sequences that have been specifically designed ("designer genes") to encode the above described synthetic muteins. Other aspects of this aspect are expression vectors that include such structural designer genes, host cells or organisms transformed with such vectors, and processes for making the synthetic mutein by culturing such transformants or their progeny and recovering the mutein from the culture. Therapeutic formulations whether for human or veterinary use (and optionally in unit dosage form) are another aspect of the invention.

Another aspect of the invention is a method of preventing a protein having one or more cysteine residues that is free to form an undesirable disulfide link from forming such a link characterized in that the protein is mutationally altered by deleting the cysteine residue(s) or replacing it/them with other amino acids.

Still another aspect of the invention is a method for making the above described synthetic structural gene by oligonucleotide-directed mutagenesis comprising the following steps:

(a) hybridizing single-stranded DNA comprising a strand of a structural gene that encodes the parent protein with a mutant oligonucleotide primer that is complementary to a region of the strand that

includes the codon for the cysteine to be deleted or replaced or the antisense triplet paired with the codon, as the case may be, except for a mismatch with that codon or antisense triplet, as the case may be, that defines a deletion of the codon or a triplet that encodes said other amino acid;

(b) extending the primer with DNA polymerase to form a mutational heteroduplex; and

(c) replicating the mutational heteroduplex.

The mutant oligonucleotide primers used in this process are another aspect of the invention.

The invention will now be further described and illustrated with reference to the accompanying drawings, in which:-

Figure 1 is a diagram of the amino acid sequence of IFN-ß;

Figure 2 is a schematic illustration showing the preparation of a mutant IFN-ß gene by oligonucleotide-directed mutagenesis;

Figure 3 shows a diagram of plasmid pß1trp including the IFN-ß gene;

Figure 4 is a diagram of the cloning vector M13mp8 phage;

Figure 5 shows the restriction map of clone M13-ß1;

Figure 6 shows the sequencing gel pattern of the mutant IFN-ß$_{ser17}$ gene showing a single base change in the coding region;

Figure 7 is a diagram of the expression plasmid pTrp3;

Figure 8a shows the HinfI restriction pattern of clone pSY2501 and Figure 8b shows the resulting two 169bp and 28bp fragments thereof;

Figure 9 is a restriction map of clone pSY2501;

Figure 10 shows the coding DNA sequence for the mutein IFN-β_{ser17} with the corresponding amino acid sequence therefor;

Figure 11 shows the single 18,000 dalton protein band corresponding to IFN-β_{ser17} in the extracts of clones pSY2501 and pßltrp.

In the case of IFN-ß, it has been reported in the literature and that both the glycosylated and unglycosylated IFNs show qualitatively similar specific activities and that, therefore, the glycosyl moieties are not involved in and do not contribute to the biological activity of IFN-ß. However, bacterially produced IFN-ß which is unglycosylated consistently exhibits quantitatively lower specific activity than native IFN-ß which is glycosylated. IFN-ß is known to have three cysteine residues at positions 17, 31 and 141. Cysteine 141 has been demonstrated by Shepard, et al, supra, to be essential for biological activity. In IFN-α, which contains four cysteine residues, there are two intramolecular -S-S- bonds: one between cys 29 and cys 138 and another between cys 1 and cys 98. Based on the homology between IFN-ß and IFN-αs cys 141 of IFN-ß could be involved in an intramolecular -S-S- bond with cys 31, leaving cys 17 free to form intermolecular crosslinks. By either deleting cys 17 or substituting it by a different amino acid, one can determine whether cys 17 is essential to biological activity, and its role in -SS- bond formation. If cys 17 is not essential for the biological activity of the protein, the resulting cys 17-deleted or cys 17-substituted protein might

exhibit specific activity close to that of native
IFN-ß and would possibly also facilitate isolation and
purification of the protein.

By the use of the oligonucleotide-directed
mutagenesis procedure with a synthetic oligonucleotide
primer that is complementary to the region of the
IFN-ß gene at the codon for cys 17 but which contains
single or multiple base changes in that codon, a
designer gene may be produced that results in cys 17
being replaced with any other amino acid of choice.
When deletion is desired the oligonucleotide primer
lacks the codon for cys 17. Conversion of cys 17 to
neutral amino acids such as glycine, valine, alanine,
leucine, isoleucine, tyrosine, phenylalanine, histi-
dine, tryptophan, serine, threonine and methionine is
the preferred approach. Serine and threonine are the
most preferred replacements because of their chemical
analogy to cysteine. When the cysteine is deleted,
the mature mutein is one amino acid shorter than the
native parent protein or the microbially produced
IFN-ß.

The size of the oligonucleotide primer is
determined by the requirement for stable hybridization
of the primer to the region of the gene in which the
mutation is to be induced, and by the limitations of
the currently available methods for synthesizing
oligonucleotides. The factors to be considered in
designing oligonucleotides for use in oligonucleotide-
directed mutagenesis (eg, overall size, size of por-
tions flanking the mutation site) are described by
Smith, M. and Gillam S., supra. In general the
overall length of the oligonucleotide will be such as
to optimize stable, unique hybridization at the muta-
tion site with the 5' and 3' extensions from the muta-
tion site being of sufficient size to avoid editing of

the mutation by the exonuclease activity of the DNA polymerase. Oligonucleotides used for mutagenesis in accordance with the present invention usually contain from about 12 to about 24 bases, preferably from about 14 to about 20 bases and still more preferably from about 15 to about 18 bases. They will usually contain at least about three bases 3' of the altered or missing codon.

The method for preparing the modified IFN-β gene broadly involves inducing a site-specific mutagenesis in the IFN-β gene at codon 17 (TGT) using a synthetic nucleotide primer which omits the codon or alters it so that it codes for another amino acid. When threonine replaces the cysteine and the primer is hybridized to the antisense strand of the IFN-β gene, the preferred nucleotide primer is GCAATTTTCAGACTCAG (underlining denotes the altered codon). When it is desirable to delete cysteine, the preferred primer is AGCAATTTTCAGCAGAAGCTCCTG, which omits the TGT codon for cys. When cysteine is replaced by serine, a 17-nucleotide primer, GCAATTTTCAGAGTCAG, which includes an AGT codon for serine is the primer of choice. The T->A transition of the first base in the cys 17 codon results in changing cysteine to serine. It must be recognized that when deletions are introduced, the proper reading frame for the DNA sequence must be maintained for expression of the desired protein.

The primer is hybridized to single-stranded phage such as M13, fd, or ∅X174 into which a strand of the IFN-β gene has been cloned. It will be appreciated that the phage may carry either the sense strand or antisense strand of the gene. When the phage carries the antisense strand the primer is identical to

the region of the sense strand that contains the codon
to be mutated except for a mismatch with that codon
that defines a deletion of the codon or a triplet that
codes for another amino acid. When the phage carries
the sense strand the primer is complementary to the
region of the sense strand that contains the codon to
be mutated except for an appropriate mismatch in the
triplet that is paired with the codon to be deleted.
Conditions that may be used in the hybridization are
described by Smith, M. and Gillam, S., supra. The
temperature will usually range between about 0°C and
70°C, more usually about 10°C to 50°C. After the
hybridization, the primer is extended on the phage DNA
by reaction with DNA polymerase I, T_4 DNA polymerase,
reverse transcriptase or other suitable DNA polymer-
ase. The resulting dsDNA is converted to closed
circular dsDNA by treatment with a DNA ligase such as
T_4 DNA ligase. DNA molecules containing single-
stranded regions may be destroyed by S1 endonuclease
treatment.

The resulting mutational heteroduplex is
then used to transform a competent host organism or
cell. Replication of the heteroduplex by the host
provides progeny from both strands. Following repli-
cation the mutant gene may be isolated from progeny of
the mutant strand, inserted into an appropriate
expression vector, and the vector used to transform a
suitable host organism or cell. Preferred vectors are
plasmids pBR322, pCR1, and variants thereof, synthetic
vectors and the like. Suitable host organisms are
E.coli, Pseudomonas, Bacillus subtilis, Bacillus
thuringiensis, various strains of yeast, Bacillus
thermophilus, animal cells such as mice, rat or
Chinese hamster ovary (CHO) cells, plant cells, animal

and plant hosts and the like. It must be recognized
that when a host of choice is transformed with the
vector, appropriate promoter-operator sequences are
also introduced in order for the mutein to be expres-
sed. Hosts may be prokaryotic or eukaryotic (proces-
ses for inserting DNA into eukaryotic cells are des-
cribed in PCT applications nos US81/00239 and
US81/00240 published 3 September 1981). E.coli and
CHO cells are the preferred hosts. The muteins
obtained in accordance with the present invention may
be glycosylated or unglycosylated depending on the
glycosylation occurring in the native parent protein
and the host organism used to produce the mutein. If
desired, unglycosylated mutein obtained when E.coli or
a Bacillus is the host organism, may be optionally
glycosylated in vitro by chemical, enzymatic and other
types of modifications known in the art.

In IFN-β, the cysteine residue at position 17
in the amino acid sequence of IFN-β, as
shown in Figure 1, is changed to serine by a T→A
transition of the first base of codon 17 of the sense
strand of the DNA sequence which codes for the mature
IFN-ß. The site-specific mutagenesis is induced using
a synthetic 17-nucleotide primer GCAATTTTCAGAGTCAG
which is identical to a seventeen nucleotide sequence
on the sense strand of IFN-ß in the region of codon 17
except for a single base mismatch at the first base of
codon 17. The mismatch is at nucleotide 12 in the
primer. It must be recognized that the genetic code
is degenerate and that many of the amino acids may be
encoded by more than one codon. The base code for
serine, for example, is six-way degenerate such that
the codons, TCT, TCG, TCC, TCA, AGT, and ACG all code
for serine. The AGT codon was chosen for the prefer-
red embodiment for convenience. Similarly, threonine

is encoded by any one of codons ACT, ACA, ACC and
ACG. It is intended that when one codon is specified
for a particular amino acid, it includes all degen-
erate codons which encode that amino acid. The 17-mer
is hybridized to single-stranded M13 phage DNA which
carries the antisense strand of the IFN-ß gene. The
oligonucleotide primer is then extended on the DNA
using DNA polymerase I Klenow fragment and the resul-
ting dsDNA is converted to closed circular DNA with T_4
ligase. Replication of the resulting mutational
heteroduplex yields clones from the DNA strand con-
taining the mismatch. Mutant clones may be identified
and screened by the appearance or disappearance of
specific restriction sites, antibiotic resistance or
sensitivity, or by other methods known in the art.
When cysteine is substituted with serine, the T->A
transition, shown in Figure 2, results in the creation
of a new HinfI restriction site in the structural

gene. The mutant clone is identified by using the
oligonucleotide primer as a probe in a hybridization
screening of the mutated phage plaques. The primer
will have a single mismatch when hybridized to the
parent but will have a perfect match when hybridized
to the mutated phage DNA, as indicated in Figure 2.
Hybridization conditions can then be devised where the
oligonucleotide primer will preferentially hybridize
to the mutated DNA but not to the parent DNA. The
newly generated HinfI site also serves as a means of
confirming the single base mutation in the IFN-ß gene.

The M13 phage DNA carrying the mutated gene
is isolated and spliced into an appropriate expression
vector, such as plasmid pTrp3, and E.coli strain MM294
is transformed with the vector. Suitable growth media
for culturing the transformants and their progeny are

known to those skilled in the art. The expressed mutein of IFN-ß is isolated, purified and characterized.

The following Examples are presented to help in the better understanding of the subject invention and for purposes of illustration only. They are not to be construed as limiting the scope of the invention.

Example 1

Cloning of the IFN-ß Gene Into M13 Vector:

The use of M13 phage vector as a source of single-stranded DNA template has been demonstrated by G.F. Temple et al, Nature (1982) 296:537-540. Plasmid pß1trp (Figure 3) containing the IFN-ß gene under control of E.coli trp promoter, was digested with the restriction enzymes HindIII and XhoII. The M13mp8 (J. Messing, "Third Cleveland Symposium on Macromolecules: Recombinant DNA," Ed. A Walton, Elsevier Press, 143-153 (1981)) replicative form (RF) DNA (Figure 4) was digested with restriction enzymes HindIII and BamHI, and mixed with the pß1trp DNA which had previously been digested with HindIII and XhoII. The mixture was then ligated with T_4 DNA ligase and the ligated DNA transformed into competent cells of E.coli strain JM 103 and plated on Xgal indicator plates (J. Messing, et al, Nucleic Acids Res (1981) 9:309-321). Plaques containing recombinant phage (white plaques) were picked, inoculated into a fresh culture of JM 103 and minipreps of RF molecules prepared from the infected cells (H.D. Birnboim and J. Doly, Nucleic Acid Res (1979) 7:1513-1523). The RF molecules were digested with various restriction enzymes to identify the clones containing the IFN-ß insert. The restriction map of one such clone

(M13-ß1) is shown in Figure 5.. Single-stranded (ss) phage DNA was prepared from clone M13-ß1 to serve as a template for site-specific mutagenesis using a synthetic oligonucleotide.

Example 2

Site-Specific Mutagenesis:

Forty picomoles of the synthetic oligonucleotide GCAATTTTCAGAGTCAG (primer) was treated with T_4 kinase in the presence of 0.1 mM adenosine triphosphate (ATP), 50 mM hydroxymethylaminomethane hydrochloride (Tris-HCl) pH 8.0, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT) and 9 units of T_4 kinase, in 50 µl at 37°C for 1 hr. The kinased primer (12 pmole) was hybridized to 5 µg of ss M13-ß1 DNA in 50 µl of a mixture containing 50 mM NaCl, 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and 10 mM ß-mercaptoethanol, by heating at 67°C for 5 min and at 42°C for 25 min. The annealed mixture was then .chilled on ice and then added to 50 µl of a reaction mixture containing 0.5 mM each of deoxynucleoside triphosphate (dNTP)', 80 mM Tris-HCl, pH 7.4, 8 mM $MgCl_2$, 100 mM NaCl, 9 units of DNA polymerase I, Klenow fragment, 0.5 mM ATP and 2 units of T_4 DNA ligase, incubated at 37°C for 3 hr and at 25°C for 2 hr. The reaction was then terminated by phenol extraction and ethanol precipitation. The DNA was dissolved in 10 mM Tris-HCl pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 50% sucrose and 0.05% bromophenylblue and electrophoresed on 0.8% agarose gel in the presence of 2 µg/ml of ethidium bromide. The DNA bands corresponding to the RF forms of. M13-ß1 were eluted from gel slices by the perchlorate method (R.W. Davis, et al, "Advanced

Bacterial Genetics", Cold Spring Harbor Laboratory,
N.Y., p. 178-179 (1980)). The eluted DNA was used to
transform competent JM 103 cells, grown overnight and
ssDNA isolated from the culture supernatant. This
ssDNA was used as a template in a second cycle of
primer extension, the gel purified RF forms of the DNA
were transformed into competent JM 103 cells, plated
onto agar plates and incubated overnight to obtain
phage plaques.

Example 3

Site Specific Mutagenesis:

The experiment of Example 2 above is
repeated except that the synthetic oligonucleotide
primer used is GCAATTTTCAGACTCAG to change codon 17 of
the IFN-ß gene from one that codes for cysteine to one
that codes for threonine.

Example 4

Site Specific Deletion:

The experiment of Example 2 above is
repeated except that the synthetic oligonucleotide
primer used is AGCAATTTTCAGCAGAAGCTCCTG to delete
codon 17 of the IFN-ß gene.

Example 5

Screening And Identification of Mutagenized Plaques:

Plates containing mutated M13-ß1 plaques
(Example 1) as well as two plates containing unmutated
M13-ß1 phage plaques, were chilled to 4°C and plaques
from each plate transferred onto two nitrocellulose
filter circles by layering a dry filter on the agar

plate for 5 min for the first filter and 15 min for
the second filter. The filters were then placed on
thick filter papers soaked in 0.2 N NaOH, 1.5 M NaCl
and 0.2% Triton X-100 for 5 min, and neutralized by
layering onto filter papers soaked with 0.5 M Tris-
HCl, pH 7.5 and 1.5 M NaCl for another 5 min. The
filters were washed in a similar fashion twice on
filters soaked in 2 x SSC (standard saline citrate),
dried and then baked in a vacuum oven at 80°C for 2
hr. The duplicate filters were prehybridized at 55°C
for 4 hr with 10 ml per filter of DNA hybridization
buffer (5 x SSC) pH 7.0, 4 x Denhardt's solution
(polyvinylpyrrolidine, ficoll and bovine serum
albumin, 1 x = 0.02% of each), 0.1% sodium dodecyl
sulfate (SDS), 50 mM sodium phosphate buffer pH 7.0
and 100 μg/ml of denatured salmon sperm DNA.
^{32}P-labeled probe was prepared by kinasing the
oligonucleotide primer with ^{32}P-labeled ATP. The
filters were hybridized to 3.5 x 10^5 cpm/ml of
^{32}P-labeled primer in 5 ml per filter of DNA hybridi-
zation buffer at 55°C for 24 hr. The filters were
washed at 55°C for 30 min each in washing buffers
containing 0.1% SDS and decreasing amounts of SSC.
The filters were washed initially with buffer contain-
ing 2 x SSC and the control filters containing unmuta-
ted M13-ß1 plaques were checked for the presence of
any radioactivity using a Geiger counter. The concen-
tration of SSC was lowered stepwise and the filters
washed until no detectable radioactivity remained on
the control filters with the unmutated M13-ß1 plaques.
The lowest concentration of SSC used was 0.1 x SSC.
The filters were air dried and autoradiographed at
-70°C for 2-3 days. 480 plaques of mutated M13-ß1 and
100 unmutated control plaques were screened with the
kinased oligonucleotide probe. None of the control

plaques hybridized with the probe while 5 mutated
M13-β1 plaques hybridized with the probe.

One of the five mutated M13-β1 plaques
(M13-SY2501) was picked and inoculated into a culture
of JM 103. ssDNA was prepared from the supernatant
and double-stranded (ds) DNA was prepared from the
cell pellet. The ssDNA was used as a template for the
dideoxy-sequencing of the clone using the M13 univer-
sal primer. The result of the sequence analysis is
shown in Figure 6, confirming that the TGT cys codon
has been converted to an AGT ser codon.

Example 6

Expression of Mutated IFN-β in E.coli:

RF DNA from M13-SY2501 was digested with
restriction enzymes HindIII and XhoII and the 520 bp
insert fragment purified on a 1% agarose gel. The
plasmid pTrp3 containing the E.coli trp promoter
(Figure 7) was digested with the enzymes HindIII and
BamHI, mixed with the purified M13-SY2501 DNA frag-
ment, and ligated in the presence of T_4 DNA ligase.
The ligated DNA was transformed into E.coli strain
MM294. Ampicillin resistant transformants were
screened for sensitivity to the drug tetracycline.
Plasmid DNA from five ampicillin resistant, tetra-
cylcine sensitive clones were digested with HinfI to
screen for the presence of the M13-SY2501 insert.
Figure 8a shows the HinfI restriction pattern of one
of the clones (pSY2501), comparing it with the HinfI
pattern of the original IFN-β clone, pβltrp. As
expected, there is an additional HinfI site in
pSY2501, cleaving the 197 bp IFN-β internal fragment
to a 169 bp fragment and a 28 bp fragment (Figure
8b). A restriction map of the clone pSY2501 is shown

in Figure 9. The complete DNA sequence of the mutant
IFN-ß gene is shown in Figure 10 together with the
predicted amino acid sequence.

The plasmid designated as clone pSY2501 was
deposited on 30 March 1933 with the Agricultural Research Culture
Collection (NRRL), Fermentation Laboratory, Northern
Regional Research Center, Science and Education

Administration, U.S. Department of Agriculture, 1815
North University Street, Peoria, Illinois 60604 and is
assigned accession numbers CMCC No. 1533 and NRRL
No. B-15356.

Cultures of pSY2501 and pßltrp, which
include progeny thereof, were grown up to an optical
density (OD_{600}) of 1.0. Cell free extracts were
prepared and the amount of IFN-ß antiviral activity
assayed on GM2767 cells in a microtiter assay.
Extracts of clone pSY2501 exhibited three to ten times
higher activity than pßltrp (Table I), indicating that
clone pSY2501 was either synthesizing more protein
exhibiting IFN-ß activity or that the protein made had
a higher specific activity.

<u>Table I</u>

EXTRACT	ANTIVIRAL ACTIVITY (U/ml)
pSY2501	6×10^5
pßltrp	1×10^5
ptrp3 (control)	30

In order to determine if clone pSY2501 was
synthesizing several times more active protein, the
extracts of both clones were electrophoresed on a SDS
polyacrylamide gel together with a control extract and
the gel stained with coomasie blue to visualize the

proteins. As shown in Figure 11, there was only one protein band corresponding to an apparent 18,000 dalton protein that was present in the extracts of clones pSY2501 and pßltrp but not in the control extract of ptrp3. This protein, which has a molecular weight of about 20,000 daltons but shows a gel migration pattern of an 18,000 dalton protein was previously shown to be IFN-ß by purification of this protein from extracts of pßltrp. Since there is less of this protein in extracts of pSY2501 than in extracts of pßltrp, the specific activity of the protein in extracts of clone pSY2501 was higher than that of clone pßltrp.

Example 7

Purification of IFN-β_{ser17}:

IFN-β_{ser17} was recovered from E.coli that had been transformed to produce IFN-β_{ser17}. The E.coli were grown in the following growth medium to an OD of 10-11 at 680 nm (dry wt 8.4 g/l).

Ingredient	Concentration
NH_4Cl	20 mM
K_2SO_4	16.1 mM
KH_2PO_4	7.8 mM
Na_2HPO_4	12.2 mM
$MgSO_4 \cdot 7H_2O$	3 mM
Na_3 citrate $\cdot 2H_2O$	1.5 mM
$MnSO_4 \cdot 4H_2O$	30 µM
$ZnSO_4 \cdot 7H_2O$	30 µM
$CuSO_4 \cdot 5H_2O$	3 µM
L-tryptophan	70 mg/l
$FeSO_4 \cdot 7H_2O$	72 µM
thiamine $\cdot HCl$	20 mg/l
glucose	40 g/l

pH control with NH_4OH

A 9.9 1 (9.9 kg) harvest of the transformed E.coli was cooled to 20°C and concentrated by passing the harvest through a cross-flow filter at an average pressure drop of ⁻110 kpa and steady-state filtrate flow rate of 260 ml/min until the filtrate weight was 8.8 kg. The concentrate (approximately one liter) was drained into a vessel and cooled to 15°C. The cells in the concentrate were then disrupted by passing the concentrate through a Manton-Gaulin homogenizer at 5°C, ⁻69,000 kpa. The homogenizer was washed with one liter phosphate buffered saline, pH 7.4 (PBS), and the wash was added to the disruptate to give a final volume of two liters. This volume was continuously centrifuged at 12000 x g at a 50 ml/min flow rate. The solid was separated from the supernatant and resuspended in four liters PBS containing 2% by wt SDS. This suspension was stirred at room temperature for 15 min after which there was no visible suspended material. The solution was then extracted with 2-butanol at a 1:1 2-butanol:solution volume ratio. The extraction was carried out in a liquid-liquid phase separator using a flow rate of 200 ml/min. The organic phase was then separated and evaporated to dryness to yield 21.3 g of protein. This was resuspended in distilled water at a 1:10 volume ratio.

The recovered product was assayed for human IFN-ß activity using an assay based on protection against viral cytopathic effect (CPE). The assay was made in microtiter plates. Fifty µl of minimum essential medium were charged into each well and 25 µl of the sample was placed in the first well and 1:3 volume dilutions were made serially into the following wells. Virus (vesicular stomatitus), cell (human fibroblast line GM-2767), and reference IFN-ß controls were

included on each plate. The reference IFN-ß used was
100 units per ml. The plates were then irradiated
with UV light for 10 min. After irradiation 100 µl of
the cell suspension (1.2 x 10^5 cells/ml) was added to
each well and the trays were incubated for 18-24 hr.
A virus solution at one plaque-forming unit per cell
was added to each well except the cell control. The
trays were then incubated until the virus control
showed 100% CPE. This normally occurred 18-24 hr
after adding the virus solution. Assay results were
interpreted in relation to the location of the 50% CPE
well of the reference IFN-ß control. From this point
the titer of interferon for all samples on the plate
was determined. The specific activity of the recov-
ered product was determined to be 5 x 10^7 U/mg.

Example 8

Acid Precipitation And Chromatographic Purification

The process of Example 7 was repeated except
that after extraction and separation of the aqueous
and organic phases and mixing of the organic phase
with PBS at a volume ratio of 3:1 the pH of the mix-
ture was lowered to about 5 by addition of glacial
acetic acid. The resulting precipitate was separated
by centrifugation at 10000-17000 x g for 15 min and
the pellet was redissolved in 10% w/v SDS, 10 mM DTT,
50 mM sodium acetate buffer, pH 5.5, and heated to
80°C for 5 min.

The solution was then applied to a Brownlee
RP-300, 10 µM, "Aquapore" column using a Beckman
gradient system. Buffer A was 0.1% trifluoroacetic
acid (TFA) in H$_2$O; buffer B was 0.1% TFA in acetoni-
trile. Detection was by ultraviolet absorbance at
280 nm. The solvent program was linear gradient of 0%

buffer B to 100% buffer B in three hr. Fractions
containing highest interferon activities were pooled
and the specific activity of the pooled interferon
preparation was determined to be 9.0×10^7 to
3.8×10^8 international units per mg protein, as
compared to about 2×10^8 U/mg for native IFN-ß.

Example 9

Biochemical Characterization of IFN-ß Ser_{17}

Amino acid compositions were determined
after 24-72 hr timed hydrolysis of 40 μg samples of
IFN in 200 μl of 5.7 N HCl, 0.1% phenol, at 108°C.
Proline and cysteine were determined in the same
fashion after performic acid oxidation; in this case,
phenol was omitted from the hydrolysis. Tryptophan
was analyzed after 24 hr hydrolysis of 400 μl samples
in 5.7 N HCl, 10% mercaptoacetic acid (no phenol).
Analysis was performed on a Beckman 121MB amino acid
analyzer using a single column of AA10 resin.

The amino acid composition calculated from
representative 24-,48-, 72-hr acid hydrolyses of
purified IFN-ß Ser_{17} agrees well with that predicted
by the DNA sequence of the clones IFN gene, minus the
missing N-terminal methionine.

The amino acid sequence of the first 58
residues from the amino acid terminus of purified IFN
was determined on a 0.7 mg sample in a Beckman 890C
sequanator with 0.1 M Quadrol buffer. PTH amino acids
were determined by reverse-phase HPLC on an Altex
ultrasphere ODS column (4.6 x 250 mm) at 45°C eluted
at 1.3 min at 40% buffer B, and 8.4 min from 40-70%
buffer B, where buffer A was 0.0115 M sodium acetate,
5% tetrahydrofuran (THF), pH 5.11 and buffer B was 10%
THF in acetonitrile.

The N-terminal amino acid sequence of IFN-ß
Ser$_{17}$ determined matches the expected sequence predic-
ted from the DNA sequence, except for the absence of
N-terminal methionine.

5   As indicated above, the IFN-ß$_{ser17}$ prepara-
tion exhibits specific activity levels very close to
or better than that of native IFN-ß. IFN-ß$_{ser17}$ has
no free sulfhydryl groups but indicates one -S-S- bond
between the only remaining cysteines at positions 31

10   and 141. The protein does not readily form oligomers
and appears to be substantially in the monomeric form.
The IFN-ß$_{ser17}$ obtained in accordance with this inven-
tion may be formulated either as a single product or
mixtures of the various forms, into pharmaceutically or

15   veterinarily acceptable preparations, e.g. in inert, non-
toxic, nonallergenic, physiologically compatible carrier
media for clinical and therapeutic uses in cancer therapy
or in conditions where interferon therapy is indicated and
for viral infections. Such formulations may be in unit

20   dosage form. Such media include but are not
limited to distilled water, physiological saline,
Ringer's solution, Hank's solution and the like.
Other nontoxic stabilizing and solubilizing additives
such as dextose, HSA (human serum albumin) and the

25   like may be optimally included. The therapeutic
formulations may be administered orally or paren-
terally such as intravenous, intramuscular, intraperi-
toneal and subcutaneous administrations. Preparations
of the modified IFN-ß of the present invention may

30   also be used for topical applications in appropriate
media normally utilized for such purposes.

          The principal advantages of the above des-
cribed mutein of IFN-ß lie in the elimination of a
free sulfhydryl group at position 17 in IFN-ß, thereby

35   forcing the protein to form correct disulfide links

between cys 31 and cys 141 and to assume the conformation ostensibly required for full biological activity. The increased specific activity of the IFN-β_{ser17} enables the use of smaller dosages in therapeutic uses. By deleting the cysteine at position 17 and eliminating the free -SH group, the IFN-β_{ser17} protein does not form dimers and oligomers so readily as the microbially produced IFN-β. This facilitates purification of the protein and enhances its stability.

CLAIMS:

1. A biologically active recombinant synthetic interferon-beta mutein in which the cysteine residue normally present at position 17, numbered in accordance with native human interferon-beta, and which is free to form a disulfide link and is non-essential to said biological activity has been depleted or replaced by a neutral amino acid.

2. A mutein as claimed in claim 1 wherein said cysteine is replaced by serine, threonine, glycine, alanine, valine, leucine, isoleucine, histidine, tryosine, phenylalanine, tryptophane or methionine.

3. A mutein as claimed in claim 1, wherein said neutral amino acid is serine.

4. A human recombinant $serine_{17}$ interferon-beta mutein.

5. A human recombinant $serine_{17}$ interferon-beta mutein which exhibits the biological activity of native human interferon-beta and which has the deduced amino acid sequence as represented in Fig. 10 with or without N-terminal methionine.

6. A mutein as claimed in any one of claims 1 to 5 and which is unglycosylated.

7. A structural gene having a DNA sequence that encodes a synthetic mutein as defined in any one of claims 1 to 6.

8. An expression vector that includes a structural gene as defined in claim 7.

9.   Plasmid pSY2501, as obtainable from deposit NRRL B-15356.

5   10.   E.coli tranformed with an expression vector as defined in claim 8 or with a plasmid as defined in claim 9.

11.   The use of an expression vector as defined
10   in claim 8 or a plasmid as defined in claim 9 or in making a synthetic recombinant interferon-beta mutein by expressing said vector or plasmid.

12.   A method of preventing an interferon-beta
15   protein having at least one cysteine residue that is free to form an incorrect disulfide link from forming said link comprising mutationally altering the protein by deleting the cysteine residue at position 17, numbered in accordance with native human
20   interferon-beta, or replacing the said cysteine residue with a neutral amino acid.

13.   A method as claimed in claim 12, wherein the cysteine residue is replaced with serine or
25   threonine.

14.   A method for making a structural gene as defined in claim 7 comprising:

30   (a)   a hybridizing single-stranded DNA comprising a strand of a structural gene that encodes for an interferon-beta protein with a mutant oligonucleotide primer that is complementary to a region of said strand that includes the codon for
35   said cysteine residue or the antisense triplet paired with said codon, as the case may be, except for a

mismatch with said codon or said antisense triplet which mismatch defines a deletion of the codon or a triplet that codes for said neutral amino acid;

(b) extending the primer with DNA polymerase to form a mutational heteroduplex; and

(c) replicating said mutational heteroduplex.

15. A method as claimed in claim 14, wherein the mismatch defines a triplet that codes for serine or threonine.

16. A method as claimed in claim 14, or claim 15, wherein the single-stranded DNA is a single-stranded phage that includes said strand and the mutational heteroduplex of step (b) is converted to a closed circular heteroduplex.

17. A method as claimed in any one of claims 14 to 16, wherein said replicating is effected by transforming a competent bacterial host with the closed circular heteroduplex and culturing the resulting transformants.

18. A method as claimed in any one of claims 14 to 17 and including the additional steps of isolating progeny of the mutant strand of the heteroduplex, isolating DNA from said progeny, and isolating said gene from the DNA from said progeny.

19. A method as claimed in any one of claims 14 to 18, wherein the protein is human interferon-beta, the cysteine residue is at position 17 and the mismatch defines a codon that codes for serine.

20. A oligonucleotide for use in making a structural gene as defined in claim 7 by oligonucleotide-directed mutagenesis and having a nucleotide sequence that is complementary to a region

5 of the strand of the structural gene that includes the codon for the cysteine residue or the antisense triplet paired with said codon, as the case may be, except for a mismatch with said codon that defines a triplet that codes for said neutral amino acid.

10

21. A process for preparing an oligonucleotide suitable for use in making a structural gene as defined in claim 7 by oligonucleotide-directed mutagenesis which comprises synthesizing by a manner

15 known per se as a nucleotide sequence that is complementary to a region of the strand of the structural gene that includes the codon for the cysteine residue or the antisense triplet paired with said codon, as the case may be, except for a mismatch

20 with said codon that defines a deleting of the codon or a triplet that codes for a neutral amino acid.

22. A process for producing a vector as defined in claim 8 comprising inserting into a vector capable

25 of transforming a host cell a gene as defined in claim 7 in a position permitting expression of said gene.

23. A pharmaceutical or veterinary formulation having interferon-beta activity, comprising a

30 synthetic mutein preparation as claimed in any one of claims 1 to 6 formulated for pharmaceutical or veterinary use, respectively, and optionally also comprising a pharmaceutically or veterinarily acceptable diluent, carrier or excipient and/or

35 optionally being in unit dosage form.

24. A formulation for use in cancer therapy, comprising:

(a) an effective amount of a recombinant human interferon-beta mutein, wherein the cystene residue at position 17, numbered in accordance with native human interferon-beta, is deleted or replaced by another amino acid and said mutein exhibits the biological activity of native, human interferon-beta;

and

(b) a non-toxic, non-allergenic, therapeutically acceptable carrier medium.

25. A formulation as claimed in claim 24, wherein the mutein is interferon-beta-serine$_{17}$ and said carried is distilled water, Ringer's solution, Hank's solution or physiological saline.

26. A formulation as claimed in claim 24 or claim 25 which includes non-toxic stabilizing and solubilizing additives selected from dextrose and human serum albumin.

27. A formulation as claimed in any one of claims 24 to 26 and in unit dosage form.

28. A formulation as claimed in any one of claims 24 to 26 and in topical application form.

29. A synthetic mutein as claimed in any one of claims 1 to 6 for use in prophylaxis, therapy or diagnosis practised on the human or animal body.

30. A method for making a pharmaceutical or veterinary formulation as defined in claim 23, which method comprises formulating for pharmaceutical or veterinary use, respectively, a synthetic mutein as defined in any one of claims 1 to 6.

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	5	10	15	20
	MetSerTyrAsnLeu	LeuGlyPheLeuGln	ArgSerSerAsnPhe	GlnCysGlnLysLeu
	25	30	35	40
	LeuTrpGlnLeuAsn	GlyArgLeuGluTyr	CysLeuLysAspArg	MetAsnPheAspIle
	45	50	55	60
	ProGluGluIleLys	GlnLeuGlnGlnPhe	GlnLysGluAspAla	AlaLeuThrIleTyr
	65	70	75	80
	GluMetLeuGlnAsn	IlePheAlaIlePhe	ArgGlnAspSerSer	SerThrGlyTrpAsn
	85	90	95	100
	GluThrIleValGlu	AsnLeuLeuAlaAsn	ValTyrHisGlnIle	AsnHisLeuLysThr
	105	110	115	120
	ValLeuGluGluLys	LeuGluLysGluAsp	PheThrArgGlyLys	LeuMetSerSerLeu
	125	130	135	140
	HisLeuLysArgTyr	TyrGlyArgIleLeu	HisTyrLeuLysAla	LysGluTyrSerHis
	145	150	155	160
	CysAlaTrpThrIle	ValArgValGluIle	LeuAgAsnPheTyr	PheIleAsnArgLeu
	165	170	175	180
	ThrGlyTyrLeuArg	Asn---		

FIG. I
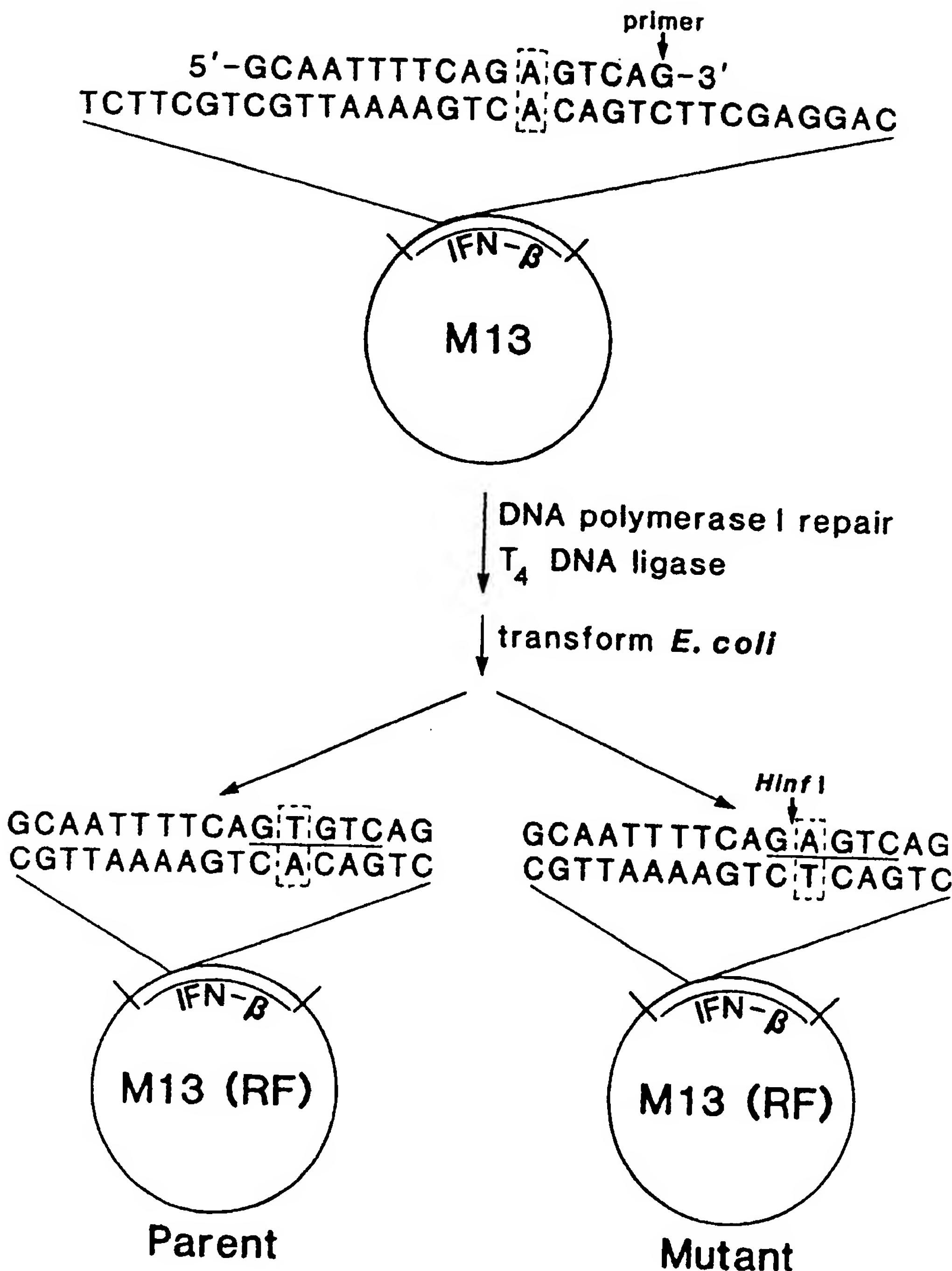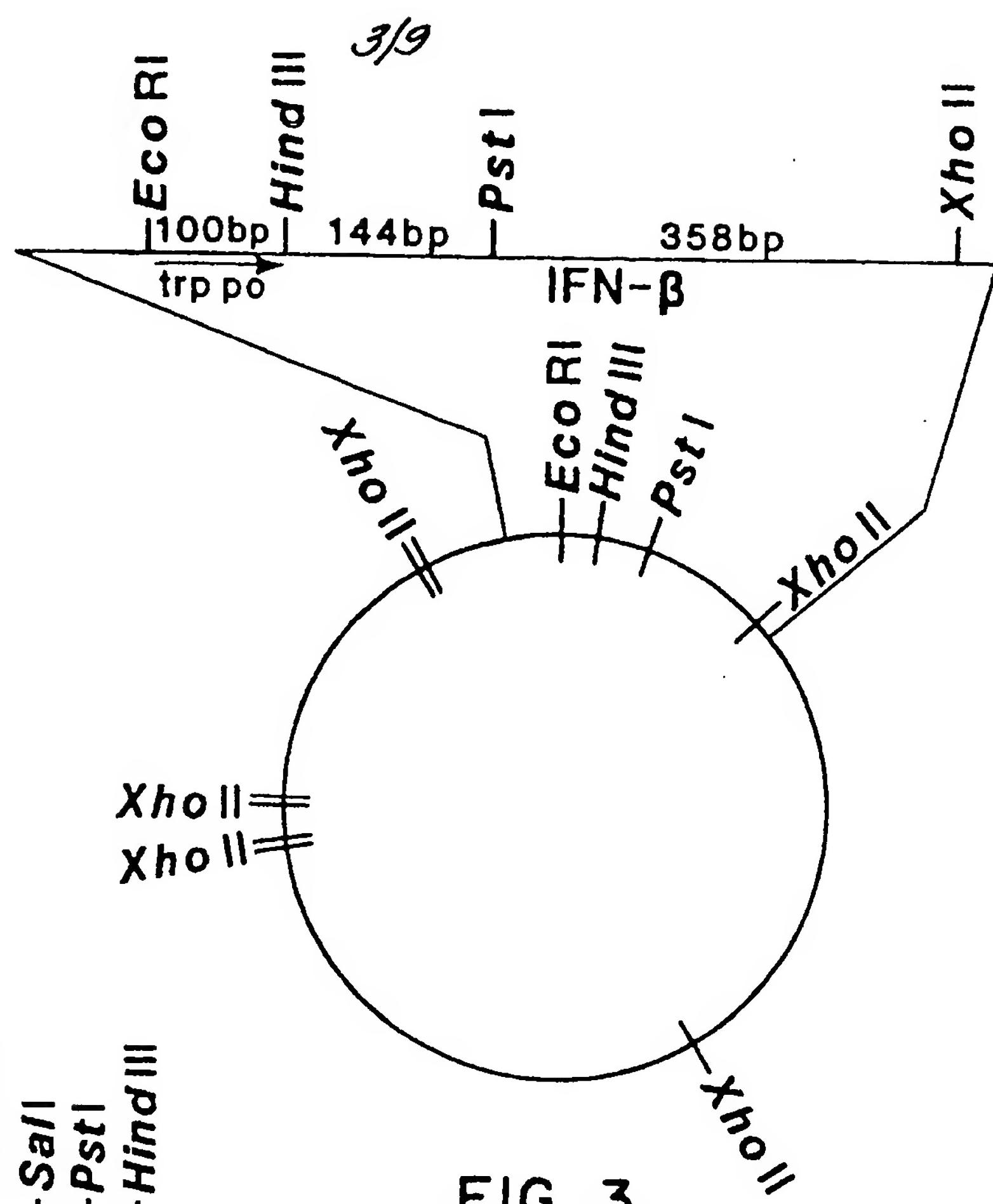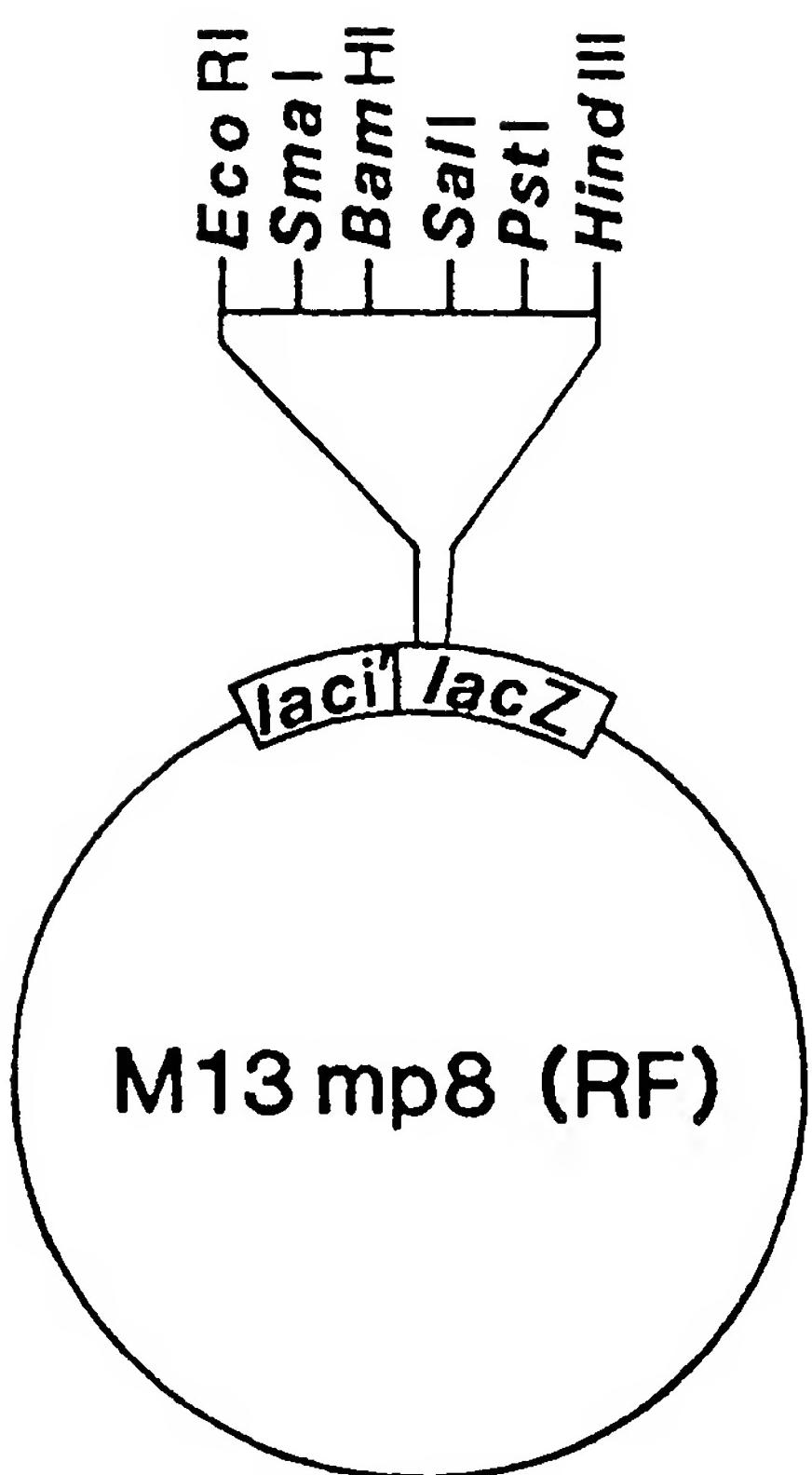
FIG. 2

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FIG. 3

FIG. 4

FIG. 5

FIG. 7

FIG. 6

FIG. 8a

FIG. 8b

EcoRI

HindIII
Hinf I
Hinf I

Pst I

Hinf I

Hinf I

Xho II

trp po

IFN-β

Xho II

EcoRI
HindIII

Pst I

Xho II

Xho II

Xho II

Xho II

FIG. 9

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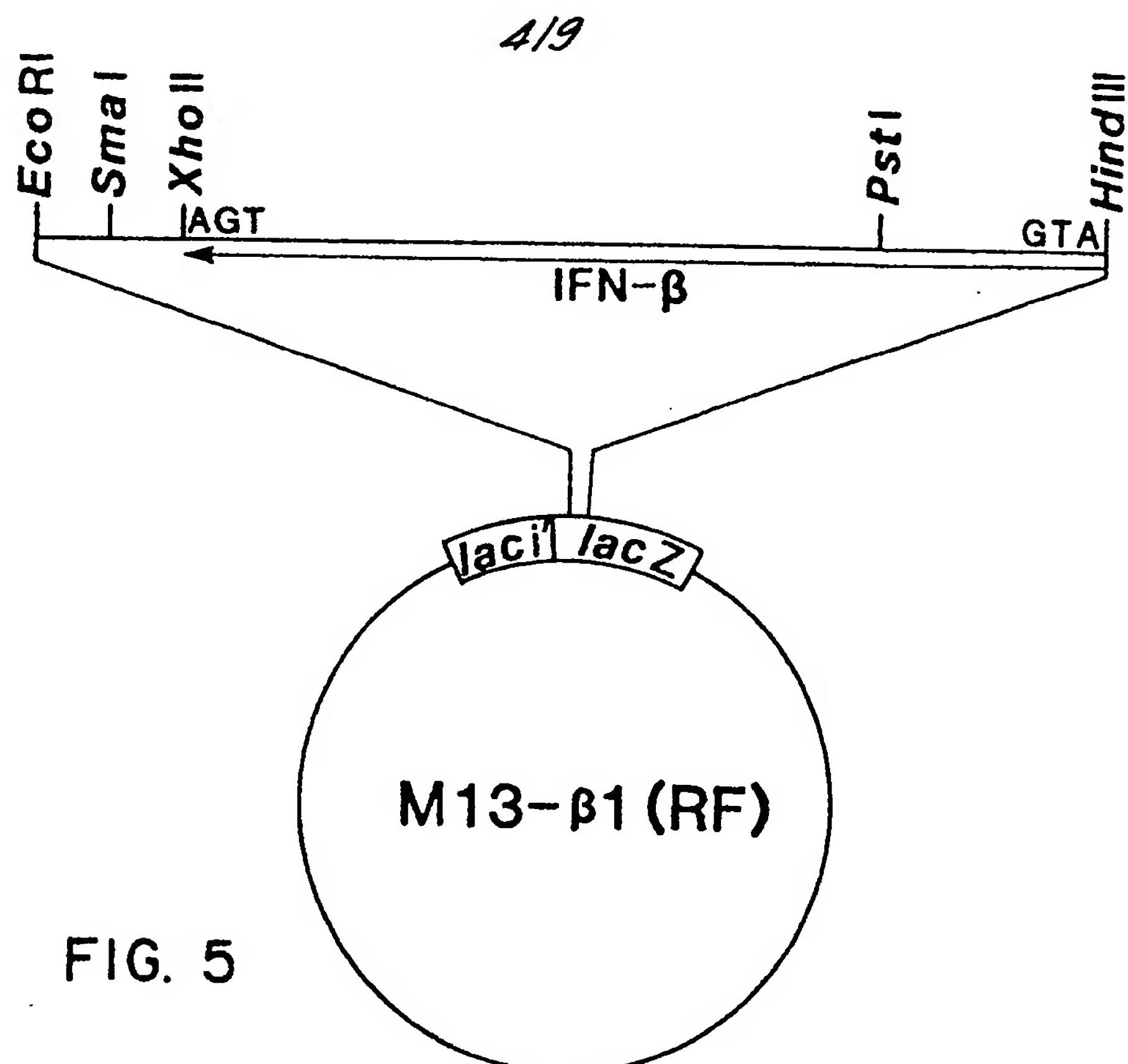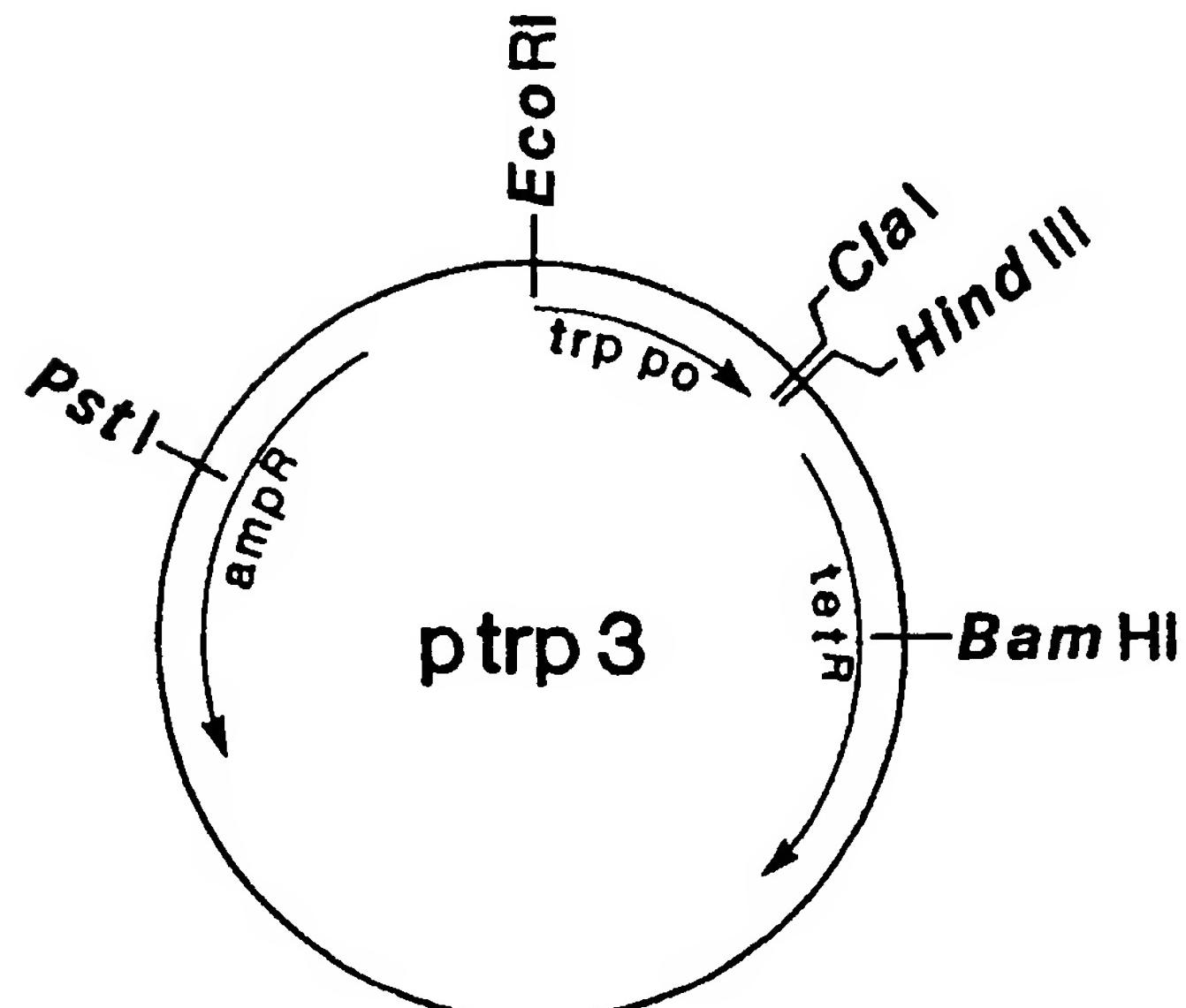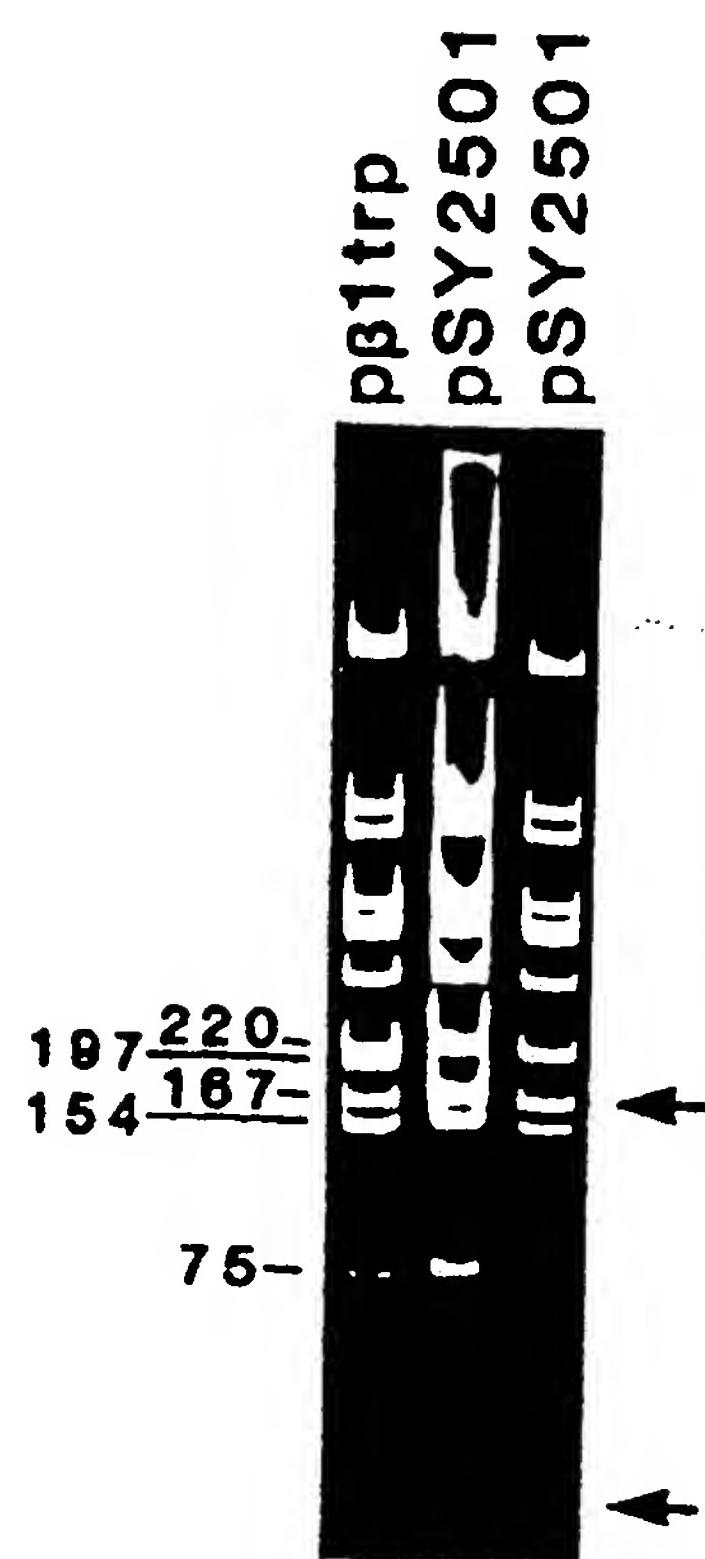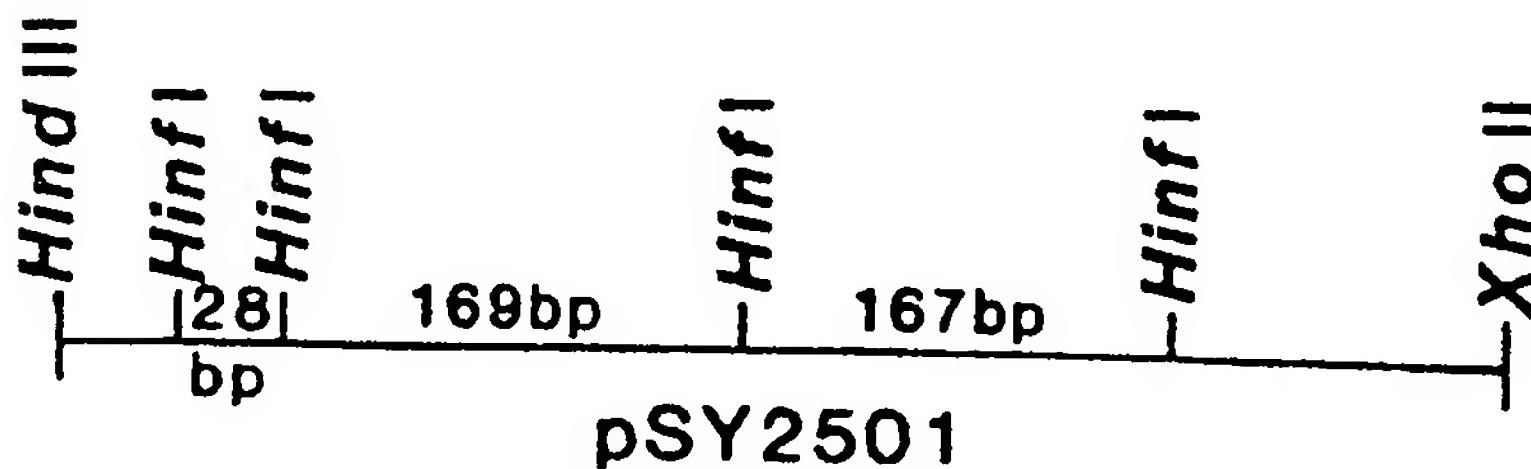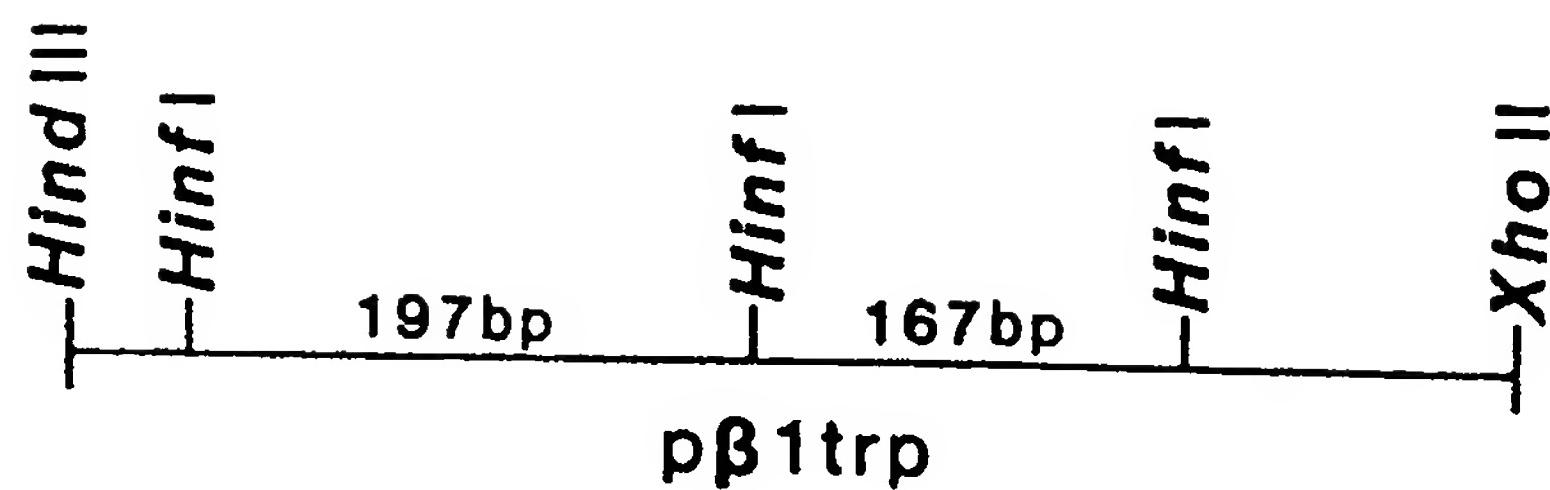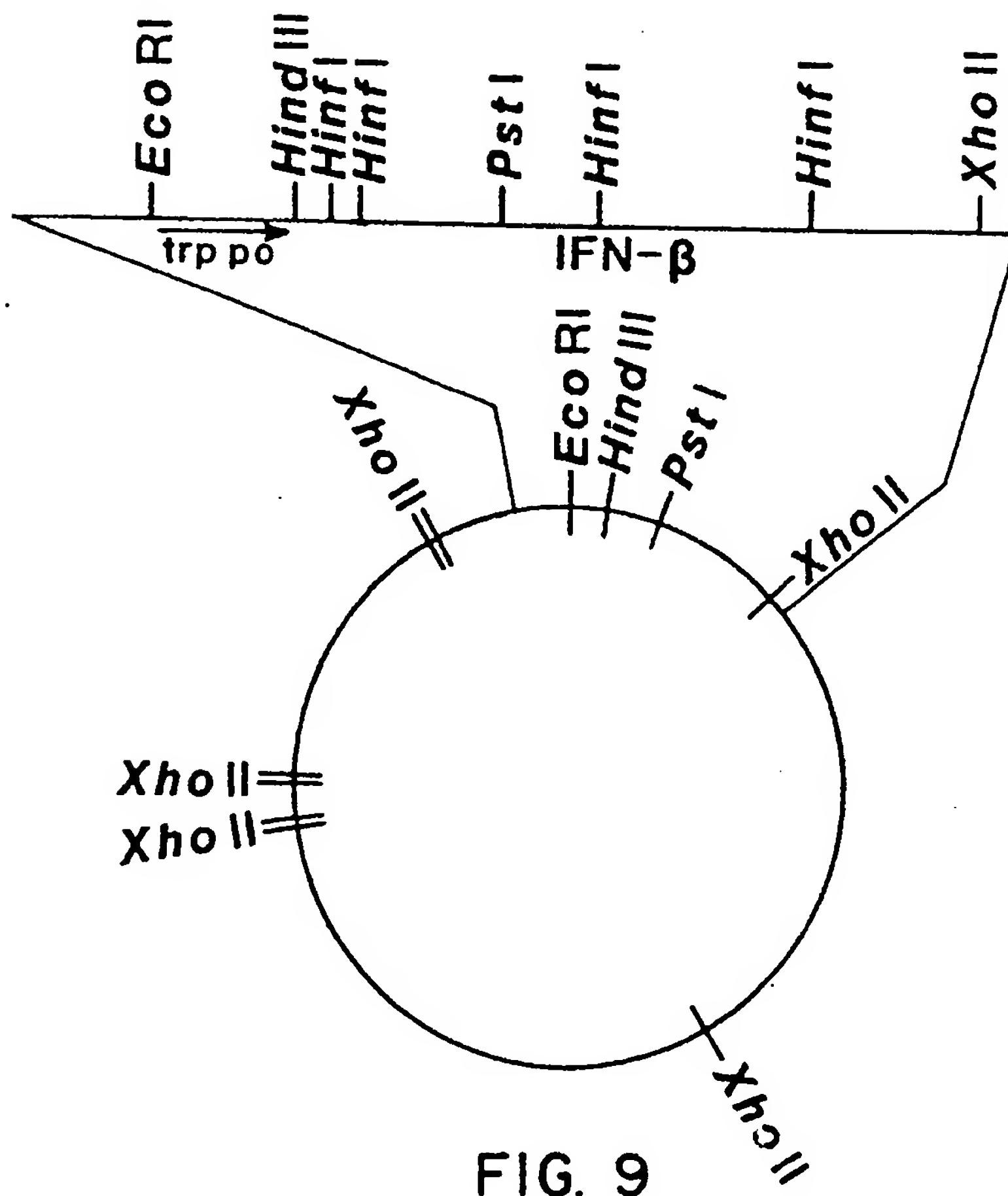
IFN-B CYS TO SER CHANGE AT AMINO ACID 17

```
1                                                             17
ATG AGC TAC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT CAG AGT CAG AAG CTC
met ser tyr asn leu leu gly phe leu gln arg ser ser asn phe gln ser gln lys leu

61
CTG TGG CAA TTG AAT GGG AGG CTT GAA TAT TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC
leu trp gln leu asn gly arg leu glu tyr cys leu lys asp arg met asn phe asp ile

121
CCT GAG GAG ATT AAG CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT
pro glu glu ile lys gln leu gln gln phe gln lys glu asp ala ala leu thr ile tyr

181
GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT AGC ACT GGC TGG AAT
glu met leu gln asn ile phe ala ile phe arg gln asp ser ser ser thr gly trp asn

241
GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA
glu thr ile val glu asn leu leu ala asn val tyr his gln ile asn his leu lys thr

301
GTC CTG GAA GAA AAA CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG
val leu glu glu lys leu glu lys glu asp phe thr arg gly lys leu met ser ser leu

361
CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC AAG GAG TAC AGT CAC
his leu lys arg tyr tyr gly arg ile leu his tyr leu lys ala lys glu tyr ser his

421
TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT
cys ala trp thr ile val arg val glu ile leu arg asn phe tyr phe ile asn arg leu

481
ACA GGT TAC CTC CGA AAC TGA AGA TC
thr gly tyr leu arg asn ***
```

FIG. 10

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FIG. II

pSY2501
pβ1trp
ptrp3

93kd
66kd

45kd

31kd

21kd
IFNβ →

14kd
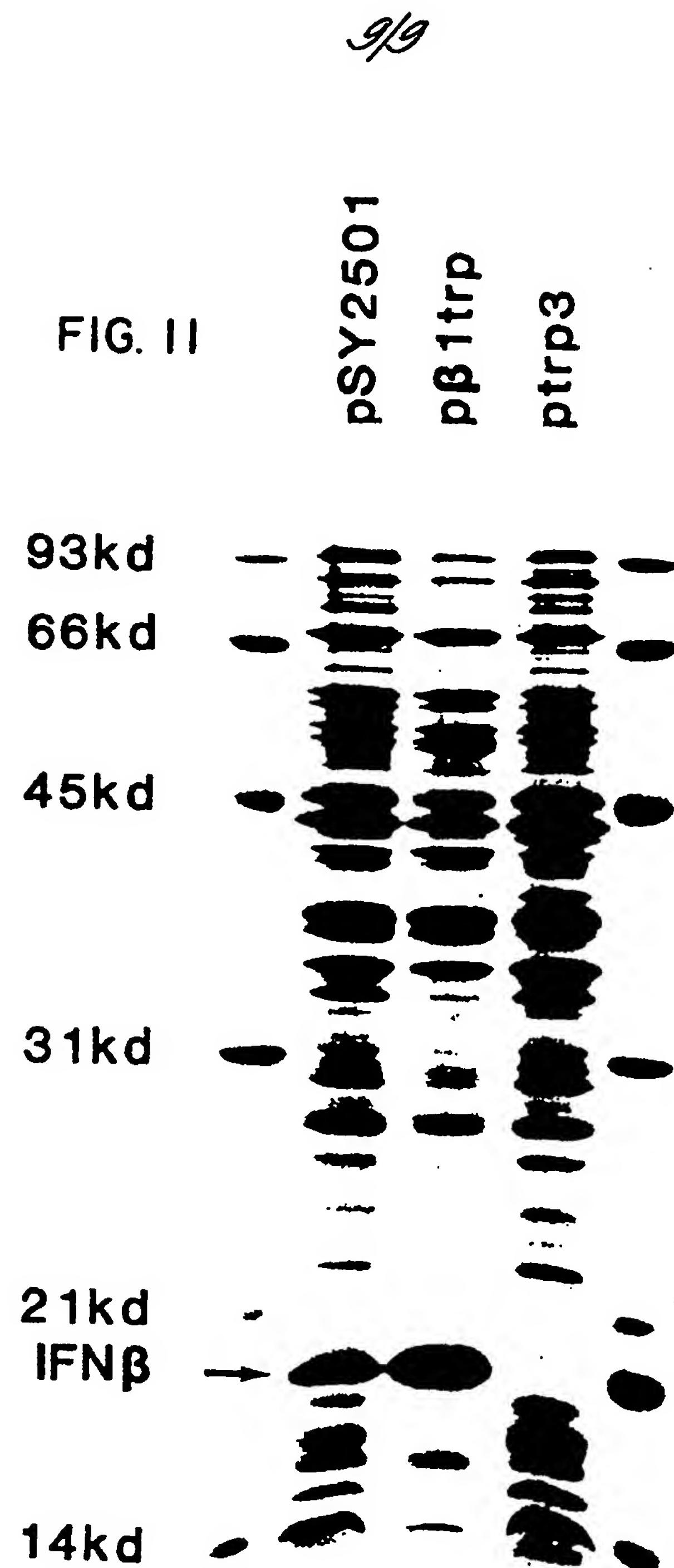
DOCUMENTS CONSIDERED TO BE RELEVANT

EP 86110595.5

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
D,A	NATURE, vol. 294, no. 5841, December 10, 1981, New York, USA H.M.SHEPARD et al. "A single amino acid change in INF-ß$_1$ abolishes its antiviral activity" pages 563-565 * Totality *	1	C 12 N 15/00 C 12 P 21/00 A 61 K 37/02 A 61 K 45/02 C 12 P 19/30 (C 12 N 15/00; C 12 R 1:19)
A	EP - A2 - 0 041 313 (BIOGEN N.V.)		

TECHNICAL FIELDS
SEARCHED (Int. Cl.4)

C 12 N
C 12 P
A 61 K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	04-12-1986	BECKER

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO Form 1503. 03.82

DECLARATION PURSUANT TO RULE 28, PARAGRAPH 4, OF THE EUROPEAN PATENT CONVENTION

The applicant has informed the European Patent Office that, until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, the availability of the micro-organism(s) identified below, referred to in paragraph 3 of Rule 28 of the European Patent Convention, shall be effected only by the issue of a sample to an expert.

IDENTIFICATION OF THE MICRO-ORGANISMS

Accession numbers of the deposits: NRRL B-15356
CMCC 1533